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Evaluation of dental adhesive systems incorporating an antibacterial monomer eugenyl methacrylate (EgMA) for endodontic restorations

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Abstract

Objectives: The purpose of this study was to incorporate EgMA, an antibacterial monomer into two commercial dental adhesives systems for their application in endodontic restoration with the aim to disinfect the root canal space before curing and to inhibit bacterial growth on their surfaces after being cured.

Methods: EgMA monomer was added at 20 % wt into the formulation of the single-component self-etch, Clearfil Universal Bond™ (CUB) and into the catalyst and the adhesive components of the total-etch Adper Scotchbond-multipurpose™ (SBMP) adhesive systems. The degree of conversion (DC) was calculated from FTIR spectra, glass transition temperature (T_g) determined by DSC, water sorption and solubility were measured gravimetrically, and surface free energy (SFE) via contact angle measurements. The bonding performance to coronal and middle root canal dentine was assessed through push-out bond strength after filling the canals with a composite core material and the surface integrity was observed using SEM and confocal laser scanning microscope (CLSM). The standard agar diffusion test (ADT) was used to identify the sensitivity of three endodontically pathogenic bacteria, *E. faecalis*, *S. mutans* and *P. acnes* to uncured EgMA modified adhesives. Multispecies biofilm model from these strains was grown on the disc surface of cured adhesives

and investigated using quantitative microbial culture and CLSM with live/dead staining. MTT assay was also used to determine the cytotoxicity of these adhesives.

Results: The incorporation of EgMA lowered polymerization exotherm and enhanced the hydrophobic character of these adhesives, without changing the DC and Tg in comparison to the controls (without EgMA). The total push-out bond strengths of the EgMA-containing adhesives were not significantly different from those of the controls ($p > 0.05$). The modification of self-etch adhesive system enhanced the bond strength in the middle region of the roots canal. SEM of debonded specimens and CLSM examination showed the integrity of the resin-dentine interfaces. For all three bacteria tested, the sizes of the inhibition zones produced by uncured EgMA modified adhesives were significantly greater ($p < 0.05$) than those of the controls. The results of biofilm inhibition tests showed less CFU for total bacteria on bonding agents with EgMA compared to the control materials ($p < 0.05$). The modification at 20 % monomer concentration had no adverse effects on cytocompatibility of both adhesives tested.

Significance: The inclusion of EgMA endows dental adhesives with effective antibacterial effects without influencing their curing properties, bonding ability to root canal dentine, and cytotoxicity against human gingival fibroblasts, indicating the usefulness of their application in endodontic restorations.

1. Introduction

Bonding of posts to root canal dentine is still a challenge due to the reduced number of dentinal tubules in the apical third of the root [1], limited access and visibility. Furthermore, the large configuration factor (C-factor) of the endodontic cavity results in a high contraction stress, that can exceed the bond strength, increasing the risk of voids and microgaps within the cement interface with subsequent bonding failure and microleakage [2]. Microleakage can cause new bacterial invasion of the root canal space also, complete removal of bacteria from the root canal system following the cleaning and shaping of the root canal is at present impossible to achieve [3]. Residual bacteria often remain in the tubules, which may repopulate the root canal and jeopardise clinical performance and longevity of the endodontic restoration. Therefore, imparting an antibacterial function to dental restorative materials, and in particular to the dental adhesives as they directly contact tooth structure and infiltrates into dentinal tubules is expected to disinfect the cavity, lowering the risk of reinfection and secondary caries.

Several attempts to produce dental adhesives with antibacterial activity have been attempted either by the addition of soluble antimicrobial agents, such as chlorhexidine, or immobilisation of antibacterial components in the resin matrix [4]. However, the release of antibacterial agent could cause an adverse effect on mechanical properties, toxicity and short-term antibacterial effectiveness whilst, the immobilisation of antimicrobial agents prevents or reduces colonisation of contacted bacteria without leaching out from the material, resulting in long-lasting antibacterial activity without adverse effects on mechanical properties and bonding characteristics [5]. A number of ionic mono and di-methacrylate monomers containing quaternary ammonium groups have been incorporated into existing dental dimethacrylate-based

monomers demonstrating bactericidal activity. For instance, Clearfil protect bond, which contains methacryloyloxydodecylpyridinium bromide (MDPB), and dental adhesives with methacryloxyethyl cetyl dimethyl ammonium chloride (DMAE-CB) have been found to exhibit an inhibitory effect on the growth of *S. mutans* [6]. However, some of the quaternary ammonium based monomers exhibit miscibility problems with hydrophobic dimethacrylates [7]. In addition, incorporation of these monomers at high concentrations to obtain reliable antibacterial effects results in adverse effects on mechanical properties and unwanted release of the monomers into the surrounding tissues [8].

Eugenol is a well-known antimicrobial essential oil, which is used in combination with zinc oxide in different dental applications such as temporary filling materials and root canal sealers and is very effective against a range of oral bacteria [9, 10]. The main disadvantage of eugenol-containing materials is the fact that they inhibit the polymerization reaction of methacrylate resins due to remaining free eugenol. Eugenyl methacrylate (EgMA) is an eugenol derivative that is able to copolymerize with other methacrylate monomers after curing and immobilises the antibacterial eugenol moieties in the polymer backbone without the inhibitory effect characteristic of the phenol group [11]. The authors have previously reported that the incorporation of an antibacterial monomer EgMA was effective in providing resin composite materials with intrinsically antibacterial activity against a range of oral bacteria commonly associated with the failure of coronal and endodontic restorations [12]. This effect based on the strong antibacterial activity of EgMA monomer [11,13]. In addition, the immobilisation of eugenol is advantageous as it avoids the migration of this molecule into the surrounding tissues and improves its hydrolytic stability.

Thus, the aim of the study was to investigate the efficacy of the modified dental adhesives via the inclusion of the eugenol methacrylate derivative. The influence of this monomer on curing properties, T_g, wettability, water sorption, bonding ability and cytotoxicity of these modified bonding agents are reported.

2. Materials and methods

2.1. EgMA incorporation into bonding agents

Two commercial adhesives, Clearfil Universal Bond™ (CUB) and Adper Scotchbond™ multi-purpose plus (SBMP) adhesives were used in this study as parent bonding systems to test the effects of incorporation of the antibacterial monomer. Their manufacturers and chemical composition are presented in Table 1. EgMA monomer was synthesized via a method reported previously by Rojo *et al* [11]. A stock solution of EgMA monomer and CQ / EDAB (0.5wt/0.5wt) both from Sigma-Aldrich (Company Ltd, Dorset, UK) was added at 20% by weight into the formulation of single-component CUB and into the adhesive/catalyst components of SBMP to prepare two modified experimental adhesives, designated respectively, as Mod.CUB and Mod.SBMP (Table 1). The selection of this percentage was based on pilot study that showed that the addition of 20 wt.% EgMA into Bis-GMA/HEMA (70/30 wt.%) blend, a commonly used dental adhesive resin formulation, had no adverse effects on degree of monomer conversion and T_g of the polymers.

2.2. Specimen preparation

For the solvated one-bottle, CUB control and modified CUB adhesives, the solvent was evaporated under reduced pressure in a dark container until the resin reached a constant mass as solvent evaporation was assumed to be complete and then carefully placed into different moulds. For total-etch SBMP control and modified

SBMP adhesives, the activator and primer were first smeared on moulds, dried with a gentle stream of air, then equal masses from adhesive and catalyst components were mixed and applied. For water sorption, solubility, surface contact angle, cytotoxicity and biofilm inhibition tests, resin discs of each material were produced in Teflon mould (10 mm diameter, 1 mm thick). After filling the mould, the discs were covered with glass slides, to exclude atmospheric oxygen, and then cured by visible light for 40 s, using a dental curing unit (Optilux, Demetron Res Crop, Danbury, USA) with an irradiance of 600 mW.cm⁻². After removing the specimen from the mould, light-curing was repeated on the opposite surface for another 40 s.

2.3. Degree of conversion

The degree of conversion of each adhesive was analysed before and after cure using FTIR spectroscopy (ATR accessory, Spectrum one, Perkin Elmer, Waltham, MA, USA). The spectra of the polymer were obtained by light-curing one drop of each adhesive between two translucent Mylar strips, pressed to produce a very thin film. Five cured specimens of each group were tested 10 min after curing and after 24h storage at 37 °C. The degree of cure was determined using equation 1

$$\text{Degree of conversion (\%)} = \left[1 - \frac{(A_{1637}/A_{1608})_{\text{polymer}}}{(A_{1637}/A_{1608})_{\text{monomer}}} \right] \times 100 \quad (\text{Eq 1})$$

Where A1637 and A1608 correspond to the absorbance of the aliphatic $\nu_{C=C}$ peak registered at 1637 cm⁻¹ and to the aromatic $\nu_{C=C}$ peak registered at 1608 cm⁻¹ respectively before and after polymerization.

2.4. Curing temperature

A thermocouple (1.3 mm diameter) fitted out to a high-sensitivity temperature recorder (KM1242, Herts, UK) was used to measure the polymerization temperature. The wire was placed centrally in a cylindrical Teflon mould (4 mm diameter, 12 mm

depth) filled with each adhesive material and its stripped ends were levelled with the material's surface to be irradiated. The materials were polymerized for 40 s from one side and the maximum temperature was reported during the polymerization cycle. Five measurements were done for each tested material at 23 °C.

2.5. Thermal properties

Differential scanning calorimetry (DSC) was carried out using a Perkin Elmer machine (Waltham, MA, USA) to determine the glass transition temperature of the cured adhesives. Samples of about 10 mg were heated from 0 °C to 230 °C at the rate of 20 °C/min in an inert N₂ atmosphere. Three samples from each formulation were tested.

2.6. Measurement of contact angle and surface free energy (SFE)

The contact angle θ and SFE (γ_s) were evaluated on bonding surface discs using the sessile drop method as described in our previous study [12].

2.7. Water sorption and solubility

Water sorption and solubility were determined according to the ISO specification 4049. Five resin discs (10 mm diameter, 1 mm thick) of each adhesive material were immersed in 10 ml distilled water and weighted at noted interval during the 28 days immersion period.

The mass change percentage was calculated using equation 4:

$$\text{Water uptake} = (M_{s(t)} - M_i) / M_i \times 100 \quad (\text{Eq 4})$$

where, M_i is the initial mass of the specimen and M_s is the mass of saturated specimen at the end of the immersion period.

The specimens were dry-stored again at 37 °C and reweighed using approximately the same time intervals until a constant dry mass (Md) was obtained.

Sorption (SR) and solubility (SL) in $\mu\text{g} / \text{mm}^3$ were calculated based on the percentage of mass gain or loss during the sorption and desorption cycles using the following equations:

$$\text{SR} = \text{Ms} - \text{Md} / V \quad (\text{Eq 5})$$

$$\text{SL} = \text{Mi} - \text{Md} / V \quad (\text{Eq 6})$$

2.8. Push-out bond strength test

Thirty-six human single-rooted premolars extracted after obtaining an informed consent of the patients and following a protocol approved by an institutional review board were used in this study (Research Ethics Committee Reference Number14/LO/0123). All teeth were stored at 4 °C in distilled water and used within one month. Teeth were randomly and equally assigned to 4 groups based on the adhesive materials used in this study (n = 9), three specimens from each group were reserved for confocal microscopy analysis. The crown was sectioned at the cemento-enamel junction using a low speed; water-cooled diamond saw microtome (Isomet 1000, Buehler, Lake Bluff, IL, USA). The teeth were endodontically treated with nickel-titanium rotary instruments (Protaper; Dentsply Maillefer, Ballaigues, Switzerland) and 1% sodium hypochlorite irrigation. The canal was filled with gutta-percha and calcium hydroxide endodontic sealer (Sealapex, Kerr, SpA, Salerno, Italy) using the lateral condensation technique. The prepared roots were mounted vertically in acrylic resin block using an aligning device. After 24 h storage at 37 °C in relative humidity, the first 8 mm of the canal was shaped with a cylindrical flat end

diamond bur (Komet 837/016, Lemgo, Germany) so that a standardised cavity of 2 mm in diameter was prepared in the coronal and middle portion of the root canal.

The dental adhesives were applied in accordance with the manufacturer's instructions. For the CUB control and modified CUB self-etch adhesives, the bonding agent was applied to the entire root canal with the applicator brush and rubbed for 10 s, then dried with a gentle air blow for 10 seconds and light-cured for 40 s using dental light-curing unit at 600 mW cm⁻² (Optilux, Demetron Res Crop, Danbury, USA). For the SBMP control and modified SBMP total-etch adhesives, the root dentine was acid-etched with 35% phosphoric acid gel for 15 s, rinsed for 20 s with water and dried with absorbent paper points (Dentsply/Maillefer, Petrópolis, RJ, Brazil). A layer of activator was applied into the root canal using disposable brushes, followed by air-drying for 5 s. The primer was applied and air-dried for 5 s. The adhesive and catalyst were mixed and applied with a fresh disposable brush and light-cured for 40 s. A dual-cure composite core (Clearfil™DC Core plus, Kuraray, Japan) was injected into the post-space and light-cured for 60s. The root segment was then placed in individually labelled containers in relative humidity at 37 °C. After 24 h, the bonded roots were transversely sectioned to create 1.5 mm thick root slices, the thickness was verified using a digital electronic calliper, the top root slices were discarded to avoid the influence of excess material, producing four root slices from each root (2 coronal and 2 middle) for subsequent push-out bond strength tests. Each slice was marked with a permanent marker on its coronal aspect and sufficiently supported by a stainless steel jig with clearance for the dislodged core material. The push-out force was applied in an apical-coronal direction using a cylindrical plunger with a diameter of 1.8 mm attached to a universal testing machine (Instron model 5569A-Series Dual Column, High Wycombe, UK) at a crosshead

speed of 0.5 mm/min until failure. The maximum load at failure was recorded in Newton (N) and was converted to MPa by dividing the applied load by the bonded area, using the following equation:

$$\text{Push-out bond strength} = \text{force} / \pi \times \text{diameter} \times \text{thickness} \quad (\text{Eq 7})$$

The modes of failure were examined visually using a stereomicroscope (WILD M32; Heerbrugg, Switzerland) at x30 and classified as adhesive failure between core material and dentine, cohesive failure with complete dentine or core material cohesive failure and mixed failure with partial interfacial adhesive failure with the presence of core/dentine cohesive failure.

Furthermore, four representative debonded specimens per group that failed in mixed or adhesive modes were selected to analyse the ultramorphology of the fractured surface with SEM. The specimens were dried overnight, mounted on aluminium stubs with carbon cement, sputter-coated with gold and observed with a scanning electron microscope (SEM, Hitachi High Technologies, S-3500N) at an accelerating voltage of 10 KeV and increasing magnifications.

2.9. Confocal laser scanning microscopy (CLSM)-Interface evaluation

For this analysis, 0.1 wt% fluorescein dye (Sigma-Aldrich, UK) was added to the single bottle component of CUB adhesives (control and modified) and to the adhesive and catalyst bottles of SBMP (control and modified) while, 0.1 wt% Rhodamine B (Rh B: Sigma- Aldrich, UK) was added into the primer bottle of Adper SBMP adhesives.

A further three specimens from each group were bonded, as previously described, with these labelled adhesive systems and employed for the confocal microscopy analysis.

After 24 h storage in 100% relative humidity, the specimens were longitudinally sectioned into two halves and polished using wet SiC abrasive papers of ascending grit #600 to #2500 (Versocit; Struers) with final ultra-sonication treatment in a distilled water bath for 5 min. The microscopy examination was performed using a confocal laser scanning microscope (Leica SP2 CLSM; Leica, Heidelberg, Germany) equipped with a 63 x /1.4 NA oil-immersion lens and using 488-nm argon/helium (fluorescein excitation) or 568-nm krypton (rhodamine excitation) laser illumination. The entire resin–dentine interface was completely investigated and three representative images of the most common distinguishing characteristics detected in each specimen were captured. All images were further reconstructed with Image J software.

2.10. Antibacterial assays

2.10.1. Agar Diffusion Test (ADT)

The antibacterial activity of uncured adhesive resins was evaluated by agar diffusion test against *E. faecalis*, *S. mutans* and *P. acnes*. The bacteria were revived from - 80 °C and plated on FAA plates (Fastidious Anaerobe Agar with 5% Horse Blood- Lab M, UK). Bacterial test suspensions with a concentration of 6×10^5 colony forming units (CFU)/mL were prepared from the pre-cultures. An aliquot of 150 µL of the bacterial suspension was spread evenly throughout the FAA agar plate using sterile swabs. Under aseptic conditions, a 20 µL portion of each bonding agent was absorbed onto sterile paper discs (6 mm diameter, 1.5 mm thick, Schleicher & Schuell, Germany) and placed on the inoculated agar surface (n=3). Pure eugenol was used as positive control. After anaerobic incubation of the plates at 37 °C for 48

h, the inhibition zones produced around the paper discs were measured [Outer diameter of inhibition zone - paper disk diameter)/2].

2.10.2. Biofilm inhibition test

Three-species biofilms composed of *E. faecalis*, *S. mutans* and *P. acnes* were grown on cured resin adhesives discs to investigate their capacity to reduce or inhibit colony formation by these bacterial species. To establish the biofilm, the bacterial strains were cultured anaerobically at 37 °C in MACS-MG-1000-anaerobic workstation (80% nitrogen, 10% hydrogen, 10% carbon dioxide) on Fastidious Anaerobe Agar supplemented with 5% defibrinated horse blood (FAA, Lab M, Heywood, UK). An individual starter culture of each bacterial strain was transferred into 3 mL of modified fluid universal medium (mFUM) and incubated anaerobically at 37 °C for 3 h. The absorbance was adjusted with fresh mFUM to 0.5 at 540 nm to obtain 10^7 cells mL⁻¹ using Labsystems iEMS Reader (MF, Basingstoke, UK).

All discs (n= 6, for each adhesive) were soaked in distilled water at 37 °C for 24 h to remove unpolymerized monomers and then sterilised by wiping with 70% ethanol in water and were exposed to UV radiation for 30 min. Discs were placed in 1 mL of mFUM contained in 24 well tray and pre-reduced in the anaerobic workstation.

The discs were then seeded with 400 µL (4×10^6 cells) of each of the three starter cultures. The biofilms were grown anaerobically with regular medium change every 24 h for the first 7 days. In order to nutritionally starve the biofilms, they were further grown anaerobically for another 7 days without medium change following the protocol previously mention by Niazi *et al.* [14]. To enumerate the numbers of bacteria in the biofilms, each disc was placed in 1 mL of BHI (Brain-Heart Infusion Broth, Lab M) and vortex for 1 min to disperse the biofilm from the surface of the

disc. After serial dilution in BHI, aliquots (100 μ L) were plated onto duplicate FAA plates and incubated anaerobically for 7 days and colonies were counted.

Two discs from each group were gently washed twice with PBS to remove non-adherent cells and stained with Live/Dead BacLight bacterial viability kit (Invitrogen, Paisley, UK) and washed again before visualisation under a Leica SP2 confocal laser scanning microscope (CSLM). The quadrants of the biofilm on resin disc were demarcated by 4 marks made at the corners of the glass bottom of the 35mm diameter Petri dishes (SLS, UK) by using a permanent marker. Biofilm structure was examined by three different areas in each quadrant of the biofilm. The mean percentages of dead (red) and live (green) biovolumes were analysed using *bioImage_L* [15].

2.11. Cytotoxicity

MTT (Methyl tetrazolium) assay was used to evaluate the cytotoxicity of adhesives with human gingival fibroblast (P8, ScienCell™ Res. Lab., UK) at 24 h and 48 h according to the International Standard ISO 10993-5. Adhesives eluents were obtained by immersing sterile disc samples in 3 ml of fibroblast medium (500 ml basal medium, 10 ml fetal bovine serum, 5 ml of fibroblast growth supplement and 5 ml of penicillin/streptomycin solution, ScienCell™, UK) within bijoux vials, which were then placed onto a roller at room temperature. The supernatants were collected at 24 and 72 hour time points and refrigerated at -20°C to be used for cytotoxicity measurements.

HGF cells were cultured at 37°C humidified atmosphere with 5% CO₂ to reach about 80% confluent, trypsinised and then seeded on a 96 well plate (100 μ L / well) at a density of 1×10^4 cells / well. The cells were incubated at 37 °C, 5% CO₂ for 24 hours

to allow for cell to attach and acclimatisation prior to addition of the test eluents. After 24 h, the fibroblast media were removed from both plates and replaced with 100µl of the leached eluents from adhesives. Untreated cells served as a negative control while positive control cells were treated with 10 % v/v ethanol solution. Each group consisted of five replicate wells. Then the plates were incubated for 24h or 48h (exposure times), after which the test eluents were removed and replaced with 100 µL of MTT (5 mg/mL PBS) for 4 h. MTT solution was then removed and 100 µL dimethyl sulfoxide (DMSO) was added to each well. The plate was shaken for 5 min and the absorbance of the purple coloured solution was measured using a UV–visible spectrophotometer plate reader at wavelength 570nm (Opsys MR, Dynex Technologies, Chantilly, VA, USA). Relative cell viability is then expressed as a percentage of untreated negative control reading. Each experiment was done in duplicates.

2.12. Statistical analysis

After analysing the normality of data distribution (Kolmogorov-Smirnov test), a Mann-Whitney (nonparametric) test or Independent t-test (for normally distributed values) was used to determine the effects of EgMA monomer addition on properties of commercial parent adhesives tested. A one-way (ANOVA) and Tukey's post hoc test were also employed for the statistical evaluation of ADT and cytotoxicity data. In all tests, the level of significance was set at $p < 0.05$.

3. Results

3.1. Curing parameters

The initial degrees of cure for both EgMA modified adhesives were significantly lower than their corresponding control [Table 2]. However, DC of these bonding agents increased when measured at 24 h post curing, reporting values which were not statistically different from the controls ($p>0.05$). The addition of EgMA into the formulations of bonding agents significantly lowered the polymerization exotherm ($p<0.05$).

3.2. Glass transition temperature (T_g)

The DSC thermograms for the bonding agents tested are shown in Fig. 1. T_g values of CUB and Mod.CUB were $78.3 \pm 1.1^{\circ}\text{C}$ and $77.1 \pm 0.4^{\circ}\text{C}$ respectively, for SBMP and Mod.SBMP were $68.5 \pm 0.8^{\circ}\text{C}$ and $67.8 \pm 0.8^{\circ}\text{C}$ respectively. Both EgMA modified bonding agents exhibited comparable T_g values to their corresponding controls which were statistically not significant ($P>0.05$).

3.3. Water sorption, solubility and wettability

The percentage water uptake during the 28-days of immersion is presented in Figure 2. All adhesives showed the greatest mass change within the 1st day of storage in water. After that period, for both CUB and Mod.CUB, a constant decrease in mass was observed until equilibrium was reached, which occurred between the 2nd and 3rd day. Conversely, SBMP and Mod.SBMP showed a continued increase of mass until equilibrium. Both eugenyl containing bonding agents showed a reduction of water sorption capacity.

Contact angle (θ) values, surface free energy (γ_s), water sorption and solubility for all bonding agents tested are summarised in Table 3. The addition of EgMA into the

bonding formulation significantly increased the contact angle measurements with both tested liquids indicating the higher hydrophobicity of bonding surfaces. Accordingly, the surface free energy calculated decreased significantly. A significant reduction in SR and SL of Mod.CUB and SR of Mod.SBMP bond was also observed ($p < 0.05$), however, no significant difference in SL of Mod.SBMP bond was found in comparison to the control.

3.4. Push-out bond strength and SEM analysis of debonded specimens

The results of the push-out bond test and the percentages of the failure modes are shown in Table 4.

The total bond strengths achieved by Mod.CUB (self-etch) and Mod.SBMP (total-etch) adhesives were statistically not significant than that of their corresponding controls (t -test, $P > 0.05$). The failure analysis was in accordance with the push-out bond strength results of both adhesive systems, the specimens failed mainly in adhesive and mixed mode; however, some specimens exhibited composite cohesive failure. Mod.CUB bond showed more cohesive failures compared to the controls. When comparing the bond strength achieved at root level using the two bonding agents (control and modified) for each adhesive system on trial, the independent t -test showed that the bonding ability of Mod.CUB in the middle region of the roots was significantly greater than that of CUB control ($P < 0.05$). In contrast, there was no such difference observed in the coronal region of the roots ($P > 0.05$). The bonding ability of SBMB and Mod.SBMP were comparable in both regions of the roots.

SEM evaluation of debonded specimens that failed in adhesive or mixed modes is shown in Figure 3. A resin-hybridised dentine surface predominantly covered by residual resin was observed, however, some exposed dentinal tubules, were evident

in the middle region of the roots for the adhesives tested. The mid-sections of the roots adhesively bonded with the modified CUB showed that resin tags formed on most dentinal tubules in comparison with the control CUB adhesive, which also explained the higher bond strengths obtained in this region (Fig. 3, B2 and A2).

The modified SBMP adhesive showed features, which were comparable to that of its corresponding control without EgMA in coronal and middle specimens, although few resin tags inside the tubules were often detected on the surface of the middle specimens which are characterised by more exposed dentinal tubules (Fig.3 D2 and C2).

3.5. CLSM-Interface evaluation

Similarly to their corresponding controls (unmodified adhesives), the CLSM assessment showed that both EgMA modified self-etch CUB and total-etch SBMP adhesives were able to create a resin diffusion zone within the coronal and middle root dentine, forming a clear hybrid layer (approximate thickness 7-9 μm) located underneath a thick adhesive layer. The presence of resin tags is also evident in both adhesives and regions (Fig 4).

3.6. Antibacterial activity

ADT results (Table 5) showed that pure eugenol and both EgMA modified resin adhesives produced clear inhibition zones against all three bacteria tested, exhibiting statistically significant differences between them following the order *P. acnes* > *S. mutans* > *E. faecalis* ($p < 0.05$). The unmodified CUB exhibited some inhibition against *P. acnes* and *E. faecalis*, whilst the unmodified SBMP produced inhibition against *P. acnes* only. However, the zone of inhibition observed for EgMA modified adhesives were significantly higher than those obtained with the controls. The inhibition of

bacterial growth noted with Mod.CUB against all species tested tended to be greater than the inhibition recorded for Mod.SBMP, however, the *t*-test showed significance in *E. faecalis* species only.

The results of biofilm inhibition test are shown in Figure 5. The means number (\pm SD) of bacteria in 14-d biofilm grown on the surface of cured resin discs of the Mod.CUB (7.20 ± 0.14) and Mod.SBMP (7.74 ± 0.21) adhesives, as log₁₀ CFU, were significantly lower than their corresponding controls CUB (8.07 ± 0.05) and SBMP (8.9 ± 0.13) adhesives. EgMA had a significant effect on the recovery of bacteria.

Figure 6 shows representative 3D volume reconstructions of biofilm sections by using *biolmage_L* [15]. The percent cell viability measurements (green biovolumes) were high for CUB (>77%) and SBMP (>90%) control adhesives compared with their corresponding modified adhesives that showed more dead (red biovolumes) cells.

3.7 Cytotoxicity

All adhesives investigated in this study showed acceptable biocompatibility at 24 h and 48 h exposure time showing a high percentage of cell viability (Fig. 7), above 70% of the negative non-toxic control (the minimum cell viability percentage below which the material has a cytotoxic potential, ISO 10993-5). The statistical analysis revealed no significant reduction in cell viability of EgMA modified adhesives comparing to their unmodified native adhesives ($P < 0.05$) at 24 h and 72 h elution times. The results also showed that among all groups, the 72 h extract of CUB group only exhibited significantly higher cytotoxicity comparing to the negative control at both exposure times. For all groups, no significant differences were found between 24 h and 48 h exposure time.

4. Discussion

4.1. Curing and thermal properties

The curing ability of a bonding system is considered to be one of the important factors for obtaining a strong and durable bond to the dentinal substrate. The degree of conversion (DC) observed 10 min post curing (Table 2) of modified bonding agents containing 20% EgMA was significantly lower, which increased when measured at 24 h with values comparable to formulations without EgMA, indicating post-curing. The initial reduction in the degree of conversion is the consequence of the bi-functional nature of acrylic and allylic double bonds in the EgMA moiety, which further confirms the participation of the monomer during the bulk polymerization [11] leading to either branching or crosslinked structures with unreacted allylic bonds from the pendant eugenyl moiety. These results indicate that addition of EgMA had no adverse effect on curing behaviour of Bis-GMA/HEMA-based bonding resin similar to the results reported previously for Bis-GMA/TEGDMA based resin systems for composites [16].

However, the DC does not give a complete characterisation of polymer structures, for this reason, the T_g of each adhesive was also measured. T_g may reflect the extent of crosslinking by which the mechanical and physical properties of a polymer are determined. Here, despite the lower T_g of EgMA homopolymer (about 95 °C) [13] in comparison with Bis-GMA, the main methacrylate derivative within the formulations of both adhesives tested, the modification with EgMA monomer resulted in similar T_g values (Fig 1). This confirms the formation of slight crosslinking or branching within the network structures which potentially increased the physical properties and stability of these bonding agents [17]. This finding was also consistent

with previous studies in which EgMA was copolymerized with other methacrylate monomers, ethyl methacrylate (EMA) [18] and 2-hydroxyethyl methacrylate (HEMA)[13].

Furthermore, the presence of acrylic and allylic double bonds in the EgMA moiety could also lower the rate of reaction resulting in lower peak temperatures. The reduction in the exothermic polymerization (about 12 °C) was significant for both Mod.CUB and Mod.SBMP adhesives, which exhibited peak temperatures of 54.0 °C and 86.6°C respectively. This constitutes an additional advantage preventing thermal damage to adjacent root dentine and periodontal ligaments whilst the polymerization occurs within the root canal system.

4.2. Water sorption, solubility and wettability

The physical and mechanical properties of adhesive polymers may be significantly altered by the effects of water uptake and elution of components, which results in polymer swelling, plasticization and catastrophic degradation of resin–dentine bonds [19]. Fluid uptake in an oral environment could lead also to bacterial harbouring and marginal discolouration.

The chemical composition of adhesive resins and their net hydrophilicity have an effect on water sorption, solubility and water diffusion in these polymers. The presence of acidic, highly polar functional groups increases the diffusion of water molecules through the polymer matrices by binding successively to the polar sites via hydrogen bonding. Another factor that can affect the extent and rate of water uptake is the cross-link density of these polymer networks, which decreases the hole-free volume for water diffusion and subsequently reduces the water permeability of the polymer. The hydrophobic nature of EgMA substituted on

methacrylates and its ability to form slightly cross-linked structures significantly increased the hydrophobicity of both modified adhesives and resulted in a significant reduction in their water sorption values. This finding was also in agreement with a previous study in which high conversion copolymers from the hydrophilic monomer HEMA and EgMA were prepared demonstrating lower sorption with increased eugenyl moieties [13].

The water sorption is usually associated with a solubility, which is measured as a loss of dry mass in samples that have been immersed in water over time. The residual unreacted monomers or oligomers may be released from swollen dental adhesives during water sorption and subsequent polymer relaxation. Here, the self-etch adhesives showed higher values of water sorption and solubility than that of total-etch adhesives (Table 3). This is probably associated with the presence of the phosphate group and residual solvent in their composition that is in agreement with the results of a water sorption study on both solvated and non-solvated commercial adhesives [20]. It has been reported that residual solvents increase the free volume of polymers and can promote water sorption even after its evaporation prior to water immersion [19]. Furthermore, using the data obtained from SFE and contact angles, it is obvious that these adhesives also presented a more hydrophilic behaviour than total-etch adhesives. More hydrophilic polymer networks permit a rapid release of unreacted monomers through nano-voids in the material because of the higher relaxation capacity [21], showing a decrease in weight within a short time of water immersion (Fig.2). Therefore, the results of the present study indicate that the incorporation of EgMA into the self-etch adhesive formulation may be clinically useful. The modified CUB one step adhesive showed a significant reduction in solubility, limiting the extraction of any unreacted components into the surrounding

environment potentially resulting in increased mechanical stability and long-term durability of resin–dentin bonds.

The wettability of the adhesive polymers was studied because of its importance in the material-medium interactions. Thus, the surface properties were evaluated by measuring the contact angle and SFE as parameters of surface hydrophobicity considering two liquids of opposite polarity, water and methylene iodine. However, the effect of SFE of substrates on bacterial adhesion has been critically discussed in the literature with no clear consensus. Bacterial adhesion is a complex phenomenon and is related to the surface energy of substrate and bacteria. It has been reported that the total interaction energy between the bacteria and the substrate linearly increases with the decrease of the surface energy [22, 23]. Taking into consideration the previous findings for experimental polymers and composite incorporating EgMA, the greater hydrophobic character of these materials, presented a lower SFE [12], improved the total interaction energy with the bacteria and resulted in a higher accessibility to the eugenyl moieties responsible for the bacteriostatic activity [13].

4.3. Push-out bond strength and interface evaluations

The results of the current study showed that irrespective of the adhesive system, the incorporation of EgMA did not adversely affect the root dentine bonding ability of the parent adhesives. The adhesive systems selected in this study were based on two different bonding strategies, self-etch and total-etch adhesive systems. They differ in composition and are usually used in association with resin cement or composites core materials to bond the endodontic restoration to root canal dentine. EgMA was successfully incorporated into the commercial adhesives with no evidence of phase separation or agglomeration. This was confirmed by the results obtained from curing and thermal characterisation discussed above and reflected the absence of any

adverse effects on their bonding characteristics. The ability of these adhesives to bond a resin composite to the coronal and middle regions of the root canals was evaluated by push-out test, which has been accepted as a reliable method for measuring the bond strength to root canal dentine [24]. In order to test solely the composite–dentin interface in an optimum standardised way, while excluding the composite-post interface, no posts were inserted into the cavities. The push-out bond strength values obtained from all adhesives tested were within the range of previous studies on root dentine adhesion using different dental adhesives with resin composite materials [25,26]. Although the push-out strength of the self-etch adhesive CUB was not significantly higher with the inclusion of EgMA, the regional bond strengths, revealed that it created a significantly higher (t-test; $p < 0.05$) bond strength in the middle region of the canal.

CUB is a single-step adhesive which combines the primer and adhesive into one bottle, contains a strongly acidic adhesive monomer, MDP enabling simultaneous demineralisation and monomer penetration into the dentine and microfiller. Since the addition of EgMA does not affect the pH value of the adhesive (data not shown), it is speculated that the same degree of demineralization of the smear layer as the control CUB is obtained for the Mod.CUB. The improvement in bond strength could be attributed to the reduction in viscosity observed after the addition of the monomer, which might enhance the infiltration of the adhesive resin into the root dentine. The low viscosity of the EgMA monomer functioned as an excellent diluent for Bis-GMA within its composition, which lowers the initial viscosity of the monomer mixture.

SBMP is an etch-and-rinse adhesive that requires surface etching and priming before its application. The EgMA modified components of this adhesive system showed chemically comparable compatibility with its water-based primer after their

application into the acid etch dentine surface and air-drying, exhibited no significant differences in push-out bond strength value in comparison to its control.

Failure mode analysis showed adhesive failure between composite and dentine and mixed failure pattern were the most common failure types for both adhesives in control and modified groups (Table 4). However, the modified CUB demonstrated higher mixed and cohesive failure indicating the superior quality of the bond. In general, bond strength to root canal dentine is lower than bond strength to coronal dentine [27]. Several studies have reported adhesive failures predominantly between post space dentine and resin cement or composite materials [28, 29]. Furthermore, the SEM indicated similar interfacial morphological features for control and modified antibacterial adhesive systems. The de-bonded specimens often presented a residual resin covering a well-hybridized dentine surface in the coronal and middle regions of the roots. The results of SEM examination were in agreement with the findings of confocal microscopy images, showing a gap-free continuous resin-dentine interface with a clear hybrid layer in both coronal and middle regions of the roots, indicating the ability of Mod.CUB and Mod.SBMP to diffuse into self-etch and acid-etch root dentine respectively. The mix between adhesive components and primer components is also clearly observed through images shown in Fig. 4C and D. Thus, it is possible to conclude from the SEM and CLSM evaluation that the incorporation of EgMA does not impair the bonding ability of the parent adhesives applied on to root canal dentine.

4.4. Antibacterial activity

The main cause of refractory endodontic infection is the persistence of bacteria in the root canal space [30]. Cavity disinfectant, such as chlorhexidine are often used by dental clinicians before application of bonding-systems as complete removal of bacteria from the root canal system is not achievable after careful cleaning and shaping [31]. However, chlorhexidine is immiscible with dental monomers and does not undergo any polymerization. The dissolution of chlorhexidine aggregates leads to the formation of a porous surface, which potentiates staining, bacterial biofilm accumulation and leading to detrimental mechanical properties of the polymers [4]. On the other hand, if adhesive systems possessed inherent antimicrobial activity it can in addition function as a cavity disinfectant agent before polymerization which reduces clinical chair time, furthermore, the cured bonding will inhibit or reduce the growth of oral and root canal bacteria on its surface by the immobilised agent.

The ADT as standard assay was used initially in this study to determine the potential antibacterial effectiveness of unpolymerized adhesive resins containing EgMA monomer against three oral bacteria. The bacterial species used are usually prevalent in primary or secondary root canal infections [32]. *S. mutans*, virulence factor include its ability to synthesise adhesive glucans and generate acids that result in the demineralisation of dental tissues, thereby initiating dental caries on tooth structure [33]. *E. faecalis* can remain viable even after chemo-mechanical preparation [34] constituting a source of refractory endodontic infections and *P. acnes* is an opportunistic pathogen responsible for a wide range of infections and inflammatory conditions [35,36].

Both Mod.CUB and Mod.SBMP produced significantly greater inhibition zones than their corresponding controls indicating that the eugenyl residue in EgMA monomer maintains the bactericide effect of eugenol and the sensitivity of the three bacteria to this monomer within their formulations. Among the selected bacteria, EgMA containing adhesives produced significantly greater inhibition zones against *P. acnes* than *S. mutans* and *E. faecalis* ($P < 0.05$). These findings are in general agreement with previously reported studies on the eugenol and eugenol containing materials effect on *E. faecalis* [37], *S. mutans* [9, 11, 38] and *P. acnes* [39].

It is important to emphasise that some inhibition zones were also observed with unmodified control adhesives, especially with CUB. This is in agreement with previous reports and could be attributed to the presence of components that are originally incorporated to promote adhesion [40] and to the lower pH environment of self-etch adhesives containing more acidic monomers MDP [41]. However, it was acknowledged that the low pH exhibited by conventional dental bonding agents is not sufficient to ensure a reliable bactericidal activity because the acidity of these adhesive can be neutralised by the buffering action of the medium [42]. In the present study, both control adhesives tested showed no inhibition against *S. mutans* which might able to survive acidic pH [43]. It is, therefore, expected that the new formulations have the potential to kill residual bacteria in the root canal space.

A multispecies *in vitro* biofilm model was successfully established on the surface of cured adhesive resin discs to evaluate their inhibition effectiveness against biofilm formation. In our previous study, the antibacterial activity of EgMA containing composites against the adherence and growth of individual species was reported [12]. Oral bacteria *in vivo* colonise on a substrate surface to form biofilms and display properties that are dramatically distinct from their planktonic analogue in term of

antimicrobial agent resistance, which makes their elimination much more difficult. The biofilm model used in this study was nutritionally stressed to mimic the environmental conditions of the root canal system following the protocol of nutrient-stressed biofilm [14]. The previous study showed that after the initial 7 days of regular medium replenishment, the fermentation of glucose results in the production of acid, lowering the pH of broth supernatant. Leaving the biofilms for 7 days in the same medium led to the complete consumption of glucose from broth supernatant; thus, leaving the biofilm bacteria starved of the depleted fermentable carbohydrates resulting in the degradation of serum proteins and release of ammonia causing an eventual rise in the pH. The biofilm communities of refractory endodontic infections are surviving the inaccessible nutrients and pH change that possibly render them resistant to the intracanal procedures of disinfection [44].

The inclusion of 20 wt% EgMA achieved a stronger effect in reducing the biofilm viability, evidenced by decreases in the number of CFUs of the total species, live biofilm volume and percentage of live bacteria (Fig. 6). The antibacterial activities of the essential oils including eugenol have been assessed and reported [37,45]. Although the mechanisms by which these compounds exert their activity are not fully understood, it is well-known that Gram-positive bacteria are more sensitive to the essential oils, as the hydrophilic cell wall structures of Gram-negative bacteria block the penetration of hydrophobic components in the cell membrane [46]. In the case of EgMA monomer, the data suggest that the presence of a conjugated double bond in its aromatic structure and the allylic side chain is behind the strong inhibitory effects of this component [13]. In addition, its activity may be further explained in terms of the alkyl substitution into the phenol nucleus. This alters the distribution ratio between the aqueous and the nonaqueous bacterial phases by reducing the surface

tension (hydrophobicity of the compounds) or altering the species selectivity [37]. Thus, the modification of the chemical structure of the eugenol molecule to obtain the α -unsaturated ester, EgMA results in a good hydrophobic balance with a certain proton exchange capacity. This together with the presence of the 4-allyl group allows the monomer to maintain the ability to alter the cytoplasmic membrane permeability with a consequent block of ionic pumps [13]. The data also indicate that the disruptive effect on cytoplasmic membranes is maintained after the polymerization reaction indicating that EgMA based materials render bioactive bacteriostatic surfaces that reduce microbial resistance and biofilm formation. The respective virulence of each species and the differences in the chemical composition and structure of the bacteria cell walls resulted in different bacterial sensitivities toward EgMA. From ADT results, *E. faecalis* is the most resistant bacterium to EgMA monomer and *P. acnes* is the least (Table 5). Further study is needed to identify their individual colony morphology on selective media with biofilm inhibition test. However, the effectiveness of EgMA modified adhesives against *E. faecalis*, which is one of the resistant bacteria to a wide range of antibiotics, offers a considerable advantage over the commercially available dental adhesives.

4.5. Cytotoxicity

Antibacterial agents whilst inhibiting bacterial growth should also be minimally cytotoxic for mammalian cells. As is known, eugenol at high concentrations can exert some toxic effects on the dental pulp [47]. Eugenol and related compounds were shown to have a high affinity for plasma membranes because of their lipid solubility, which could contribute to cell damage [48]. However, the cytotoxicity of EgMA monomer and methacrylate polymer matrices containing EgMA with human

fibroblast has been reported earlier to show good cytocompatibility [11]. The cytocompatibility of EgMA modified adhesives exhibited HGF viability matching that of a commercial non-antibacterial control, and that of the negative control without any resin eluent. Interestingly, the Clearfil Universal Bond™ extract at 72 h exhibited significantly higher cytotoxicity compared to that at 24 h elution time and to all other groups. The acidity of the extract, which contains the acidic monomer MDP in the self-etch adhesive, may lower the pH, thus reducing cell viability significantly. However, this difference was not found in Mod.CUB group suggesting that the ability of the monomer to form a slightly cross-linked network, which limited the amount of possible leaching of the acidic monomer.

5. Conclusions

The EgMA modified self-etch and total etch dental adhesives showed antibacterial activity before and after curing against a range of endodontically pathogenic bacteria and produced an effective bond to root canal dentine and high compatibility *in vitro*, indicating a potential application to achieve successful post-endodontic restorations.

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Table 1- The chemical compositions of bonding agents tested in this study.

Boding agent	Manufacturers (patch number)	Code	Composition
Clearfil Universal Bond™ (Self-etch)	Kuraray, Tokyo, Japan (1562R041R)	CUB	MDP, Bis-GMA, HEMA, hydrophilic aliphatic dimethacrylate, colloidal silica, silane coupling agent, CQ, ethanol, water
Modified Clearfil Universal Bond™		Mod.CUB	CBU + 20 wt.% stock solution of EgMA*
Adper Scotchbond™ multi-purpose plus (Total-etch)	3M ESPE, St. Paul, MN, USA (N662538)	SBMP	Etchant: 35% Phosphoric acid gel Activator: Ethyl alcohol, sodium benzenesulfinate Primer: Water, HEMA, copolymer of acrylic, itaconic acids Adhesive: Bis-GMA, HEMA, Tertiary amines, photi-initiator Catalyst: Bis-GMA, HEMA, Benzoyl peroxide
Modified Adper Scotchbond™ multi-purpose plus		Mod.SBMP	SBMP Etchant SBMP Activator SBMP Primer SBMP Adhesive + 20 wt.% stock solution of EgMA* SBMP Catalyst + 20 wt.% stock solution of EgMA*
Abbreviations: MDP=10-Methacryloyloxydecyl dihydrogen phosphate; Bis-GMA = 2, 2-Bis [4- (2-hydroxy-3 methacryloyloxypropyl)-phenyl] propane; HEMA = hydroxyethyl-methacrylate; EgMA = eugenyl methacrylate; CQ = camphoroquinone ; EDAB = 4-(dimethylamino) benzoate. *Stock solution = [EgMA monomer + CQ / EDAB (0.5wt/0.5wt)].			

Table 2- Degree of conversion (10 minutes and 24 h post curing) and polymerization exotherm of the studied bonding agents (mean [SD], n=5).

Bonding agent	DC in % 10 min post cure [SD]	DC in % 24 h storage at 37 °C [SD]	Polymerization exotherm °C [SD]
CUB	91 [0.9]	95 [1.7]	66.8 [1.8]
Mod.CUB	88 [0.8]*	92 [1.9]	54.0 [1.8]*
SBMP	74 [0.5]	76 [1.0]	98.9 [1.7]
Mod.SBMP	66 [1.0]*	74 [1.5]	86.6 [1.5]*
* Differences were statistically significant with respect to the corresponding unmodified bonding agent (p < 0.05).			

Table 3 - Contact angle, solid surface free energy, water sorption (SR) and solubility (SL) for the bonding agents studied.

Composites	θ (H ₂ O) [S.D. ⁿ]	θ (CH ₂ I ₂) [S.D. ⁿ]	γ_s (mN/m) [S.D. ⁿ]	SR (μ g/mm ³) [S.D. ^m]	SL (μ g/mm ³) [S.D. ^m]
CUB	58.5 [3.7]	38.7 [2.2]	53.7 [2.0]	126.8 [2.1]	89.2 [1.4]
Mod. CUB	62.8 [4.0]*	41.5 [1.8]*	50.5 [2.0]*	95.1 [0.2]*	79.2 [0.6]*
SBMP	64.4 [5.7]	42.2 [5.8]	49.6 [3.2]	80.8 [1.6]	3.8 [0.5]
Mod. SBMP	69.1 [2.4]*	46.2 [4.8]*	45.6 [1.6]*	51.4 [0.4]*	3.7 [0.9]
S.D.= standard deviation, $n = 10$; $m = 5$; θ (H ₂ O)= water contact angle; θ (CH ₂ I ₂) = methylene iodide contact angle. * Differences were statistically significant with respect to the corresponding unmodified bonding agent ($p < 0.05$).					

Table 4 - Comparison of the mean push-out bond strength (MPa) values and the analysis of failure modes.

Bonding agents	Bond strength values (mean [SD])			Total failure mode (%)		
	Regional BS (n=12)		Total BS (n=24)	Adhesive	Mixed	Cohesive
	Coronal	Middle				
CUB	11.3 [2.3]	6.2 [2.0]	8.8 [3.3]	62	30	8
Mod.CUB	12.4 [1.5]	8.2 [2.4]*	10.3 [2.9]	54	34	12
SBMP	12.1 [2.0]	6.9 [0.7]	9.5 [3.0]	54	38	8
Mod.SBMP	11.6 [2.1]	6.2 [1.1]	8.94 [3.2]	58	34	8
* Differences were statistically significant with respect to the corresponding unmodified bonding agent (p < 0.05).						

Table 5 - Size of inhibition zones produced against *S. mutans*, *E. faecalis* and *P. acnes*

Group	Inhibition zone size in mm, mean [SD] of three replicates		
	<i>S. mutans</i>	<i>E. faecalis</i>	<i>P. acnes</i>
Eugenol	13.4 [0.6]	7.6 [0.6]	21.0 [1.2]
CUB	0	2.5 [0.5]	6.4 [0.6]
Mod.CUB	16.3 [1.1]*	9.5 [0.5]*	21.0 [1.1]*
SBMP	0	0	5.8 [0.7]
Mod.SBMP	14.8 [0.7]*	6.5 [1.3]*	19.1 [1.0]*
* Differences were statistically significant with respect to the corresponding unmodified bonding agent (p < 0.05).			

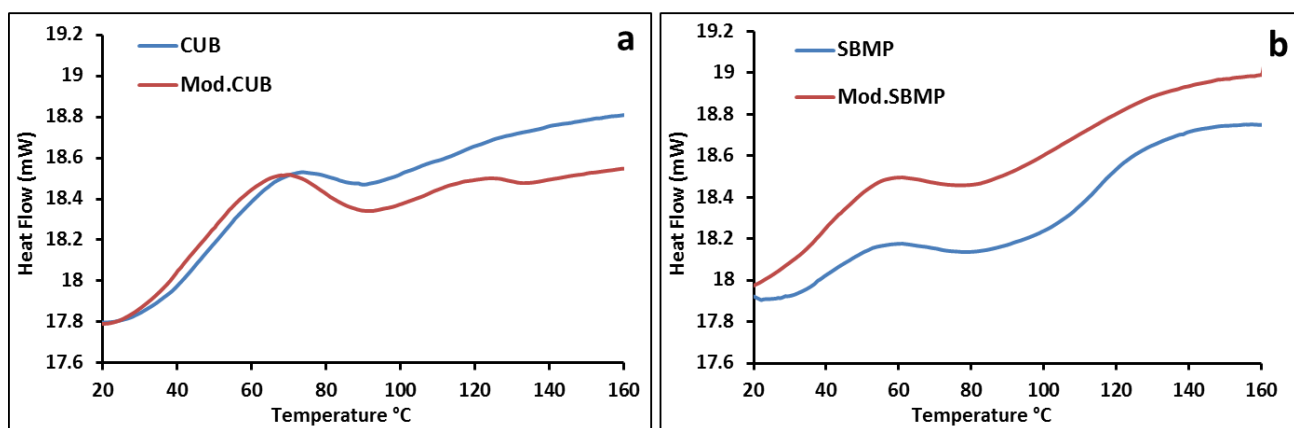


Fig. 1 - DSC representative curves of (a) CUB and Mod.CUB adhesives and (b) SBMP and Mod.SBMP adhesives.

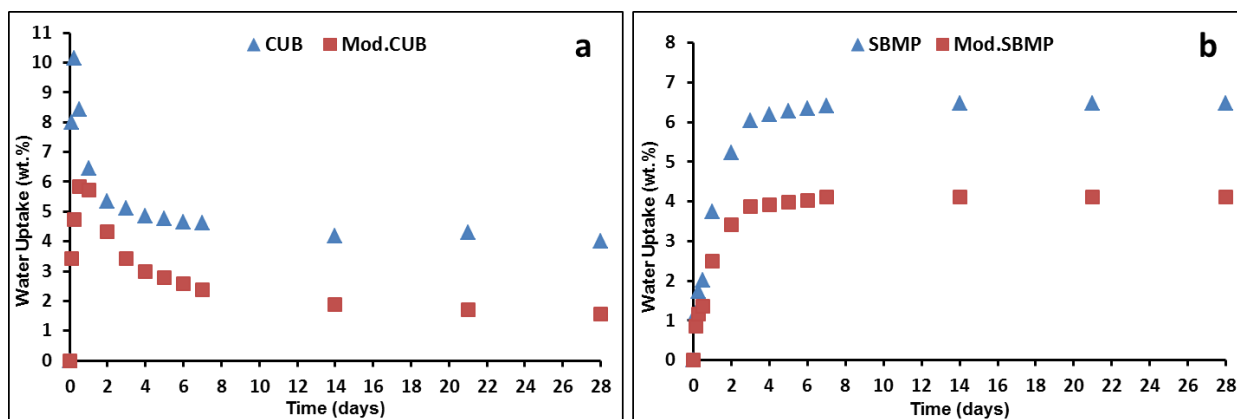


Fig. 2 - Mass changes in percentage of the bonding agents during immersion in water over 28 days.

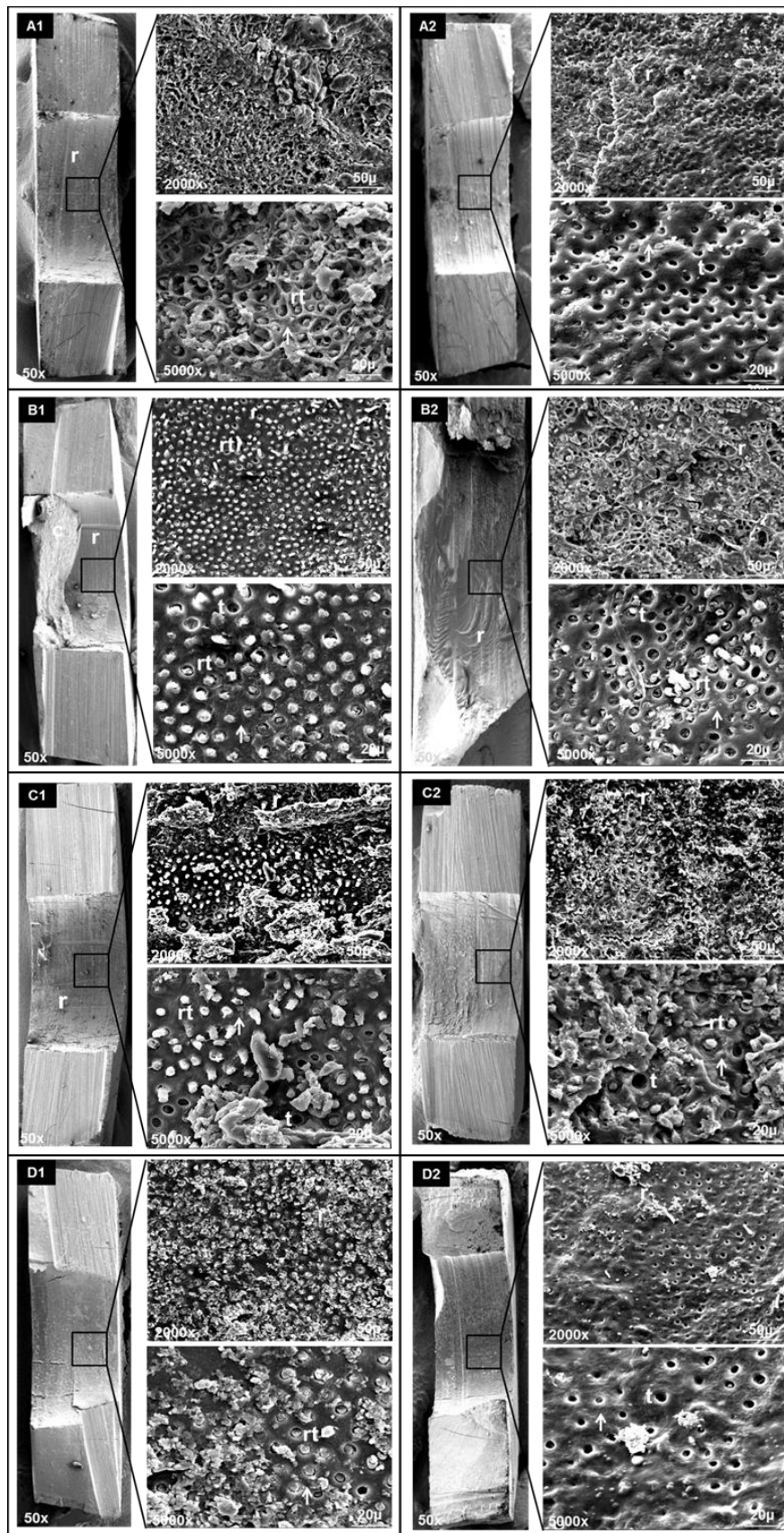


Fig. 3 - SEM images of debonded specimens created using the modified adhesives tested in comparison with their control, taken from coronal (1) and middle (2) regions at different magnifications showing the features of failure in the adhesive layer.

(A1) and (A2) are representative images of the coronal and middle sections respectively, created with unmodified CUB adhesive, which failed in adhesive mode leaving a residue of resin adhesive (r). At higher magnification shows that the residual resin blocked the dentinal tubules completely or partially and exposed dentinal tubules (t) which were more evident in the middle region (A2).

(B1) and (B2), representative images of coronal and middle sections created with Mod.CUB adhesive, (B1) coronal specimen failed in mixed mode leaving a presence of residual resin adhesive (r) and composites (c), (B2) mid-section failed in adhesive mode leaving residual resin adhesive. At higher magnification, some exposed dentinal tubules (t) are visible but most remained obliterated by resin tags (rt), of which some tags were protruding from the debonded surface and fewer number of exposed dentinal tubules were observed in the middle region in comparison with CUB adhesive. For both adhesives, a well formed hybrid layer was present on dentin surface (arrows).

(C1) and (C2) show representative images of the coronal and middle sections respectively, created with unmodified SBMP adhesive, which failed in adhesive mode showing an important presence of residual resin (r) and exposed dentin with a well formed hybrid layer (arrows). At higher magnification, it is possible to observe most of the dentinal tubules were obliterated with resin tags (rt) with few exposed tubules (t). Images from modified SBMP adhesive (D1) coronal and (D2) middle specimens (in adhesive mode) showed comparable features after failure. It is possible to see also a well-formed hybrid layer (arrow) and residual resin (r). A few resin tags inside the dentinal tubules were observed on the dentin surface of the middle specimen (D2). r, resin; t, dentinal tubules; rt, resin tags; c, composite.

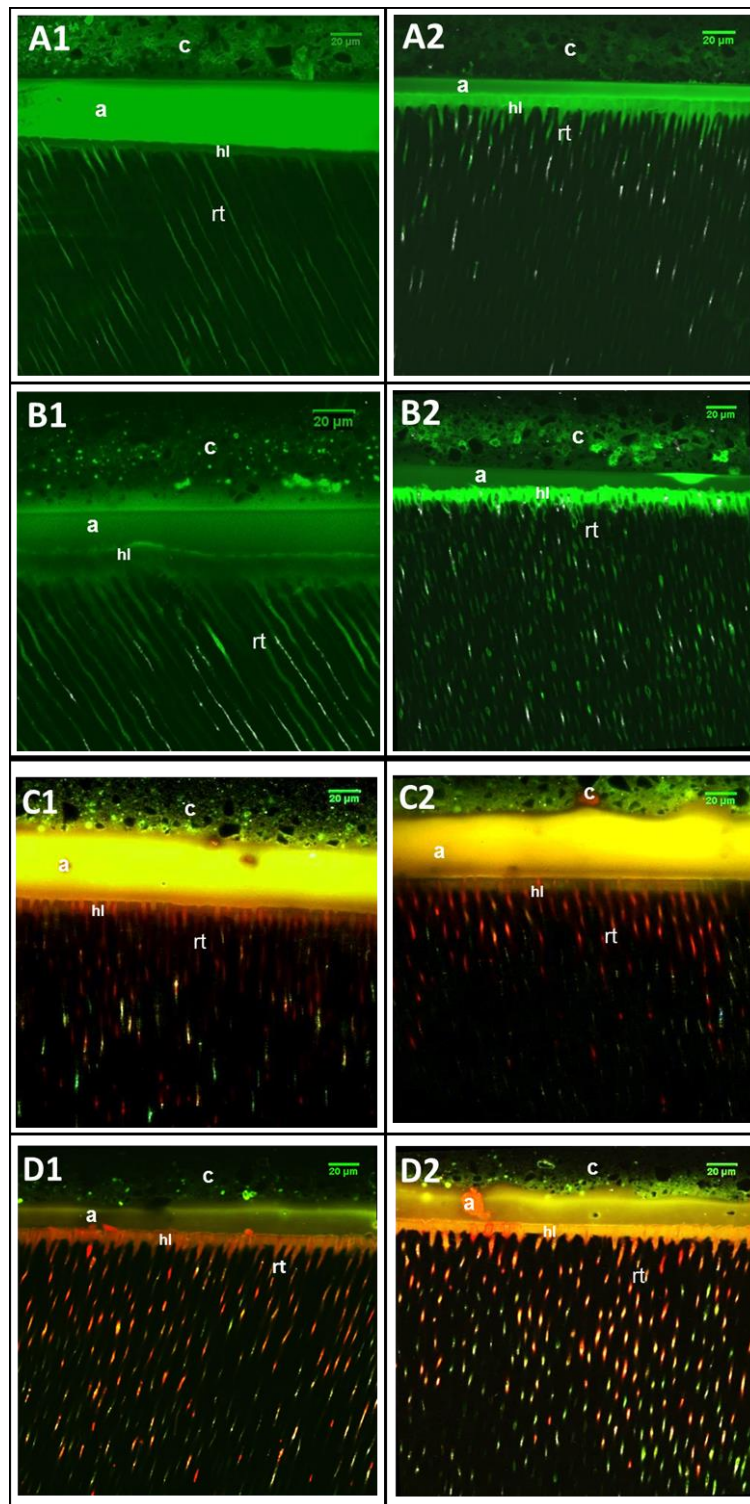


Fig. 4 - Confocal laser scanning microscopy (CLSM) images showing the interface of the resin–dentin created using the four adhesives tested from coronal (1) and middle (2) regions of the root after 24h storage in 100% relative humidity.

(A) and (B) CLSM images of the resin–dentin interfaces, taken from the coronal (1) and middle (2) regions of the roots, created with the CUB and Mod.CUB adhesives, respectively, which were labelled with fluorescein dye (green). CLSM (fluorescence/reflection mode) images show that both control unmodified and EgMA modified CUB adhesives were able to diffuse into root coronal (A1 and B1, respectively) and middle (A2 and B2, respectively) dentin, creating a gap-free interface and a clear hybrid layer (approximate thickness 7-9 μm) located underneath a thick adhesive layer and longer resin tags. (C) and (D) CLSM images of the resin–dentin interfaces, taken from the coronal (1) and middle (2) regions of the roots, created with the SBMP and Mod.SBMP adhesive systems, respectively. The primer was labelled with rhodamine B and the adhesive was labelled with fluorescein, showing red and green fluorescence colours, respectively. CLSM (fluorescence/reflection mode) composite images demonstrate an orange colour interface, which corresponded to the mixture between the primer and the adhesive components, indicating the ability of the adhesive components in both control and EgMA modified SBMP systems to diffuse into acid-etched root coronal (C1 and D1, respectively) and middle (C2 and D2, respectively) dentin, creating a gap-free interface and a clear hybrid layer (approximate thickness 7-9 μm). Thicker adhesive layers were observed in control adhesive than in modified SBMP adhesive. Resin tags are also seen in both adhesives and regions. a, adhesive layer; c, composite; hl, hybrid layer; rt, resin tags.

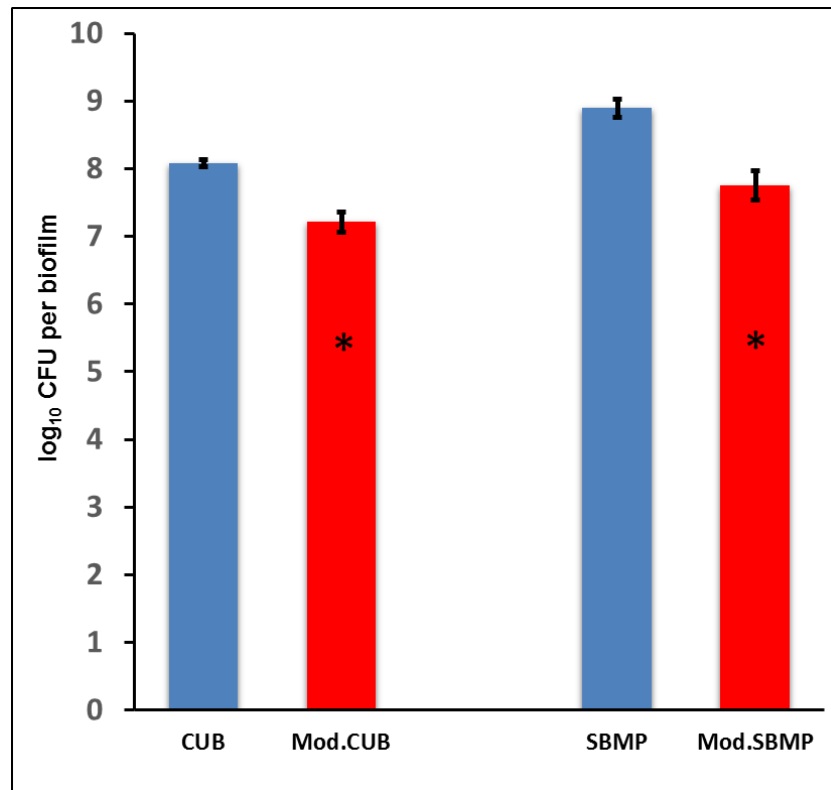


Figure 5- Colony-forming unit (CFU) counts for total microorganisms in 14-d biofilm on cured resin adhesives with and without EgMA (mean and SD; n = 4). *Differences were statistically significant with respect to the corresponding unmodified bonding agent ($p < 0.05$).

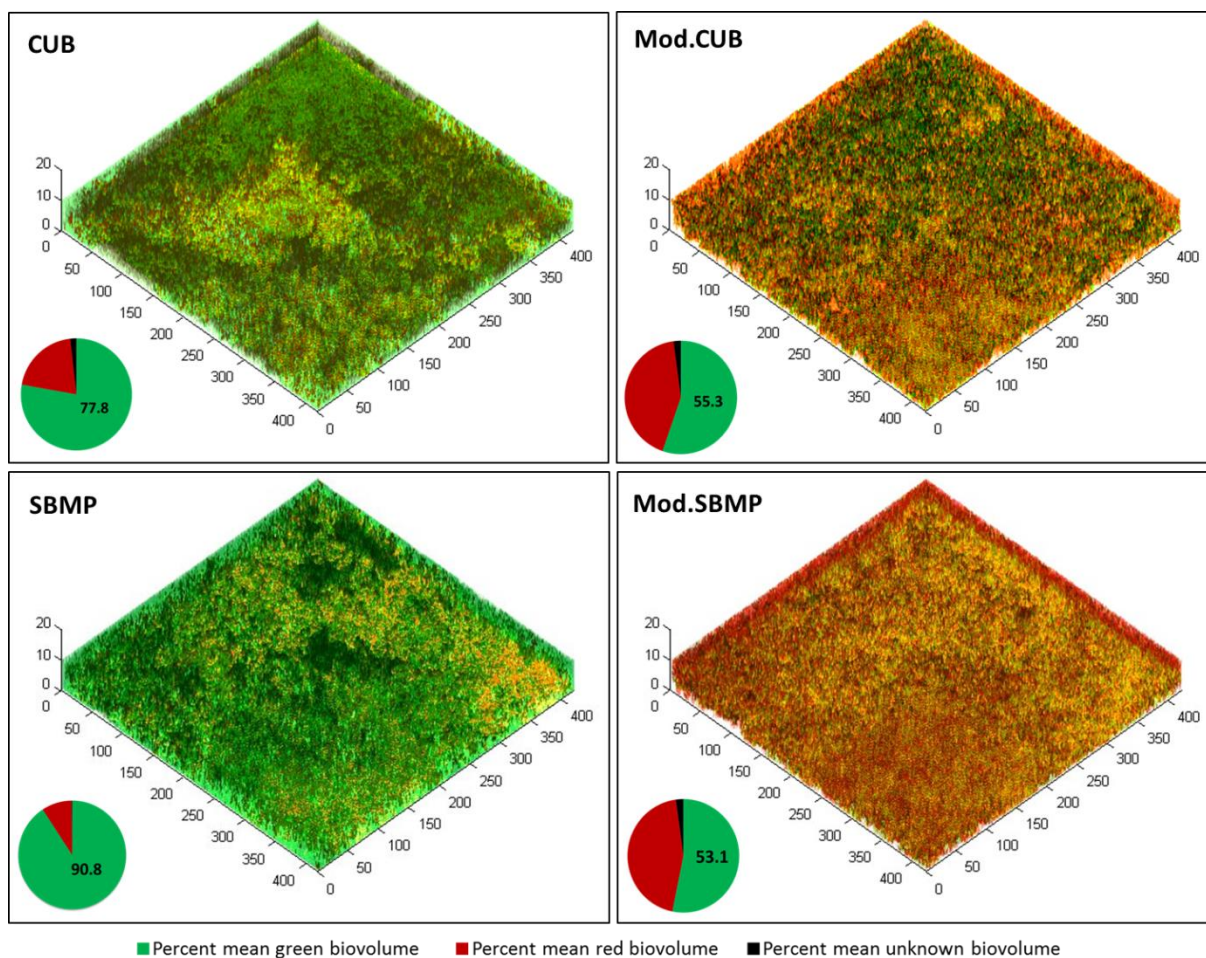


Figure 6 Biolmage L analysis results: Representative 3-D images of 14 days microbial biofilms grown on cured resin discs of the four adhesives tested with live/dead staining. The pie charts show the effect of EgMA incorporation on the mean percentages of dead (red), live (green) and unknown (black) biovolumes.

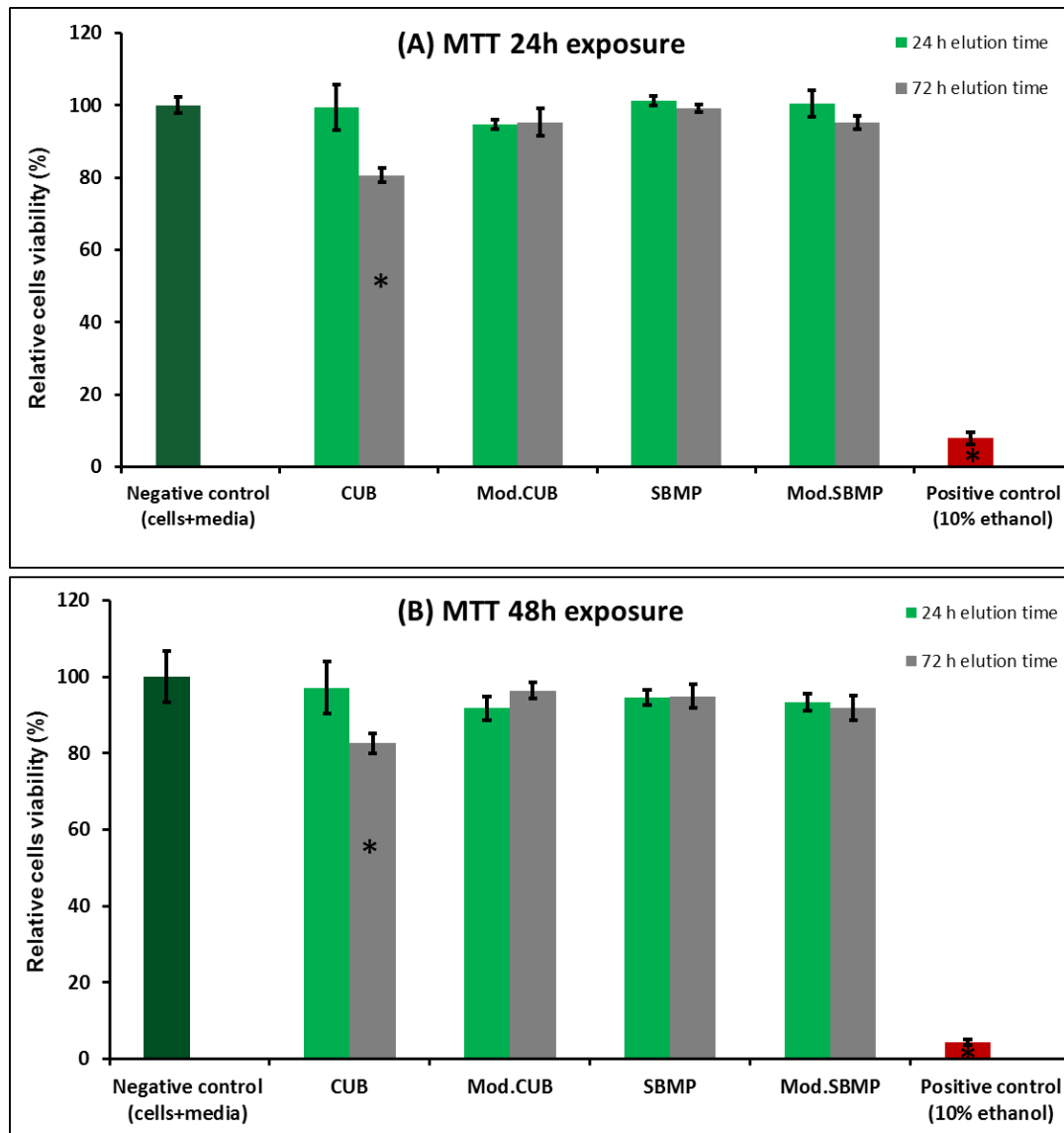


Figure 7- Viability of human gingival fibroblast (HGF) cells following exposure for (A) 24h and (B) 48h detected at 24-h and 72-h eluted media from EgMA modified and unmodified adhesives tested. The relative cell viability is presented as a percentage of the negative non-toxic control group (n=5). *Denotes significant difference when compared with negative control ($P < 0.05$).